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13. ABSTRACT (Maximum 200 Words)

Resistance to antiestrogens is a serious clinical problem in breast cancer treatment, and a better understanding of the mechanisms of antiestrogen resistance is urgently needed. Our **hypothesis**, which is supported by our preliminary data, is that the signaling molecule Cas has an important causal role in the development of antiestrogen resistance. As a corollary, understanding of the pathways that Cas activates may identify key regulators of antiestrogen resistance and novel targets for breast cancer treatment, and measurements of Cas signaling levels may provide useful prognostic information for breast cancer patients. Our **objective** is to test our hypothesis, and to identify the signaling pathways that mediate Cas-induced antiestrogen resistance. Our working model is that the Rac-JNK pathway forms a common pathway downstream of the Cas/Crk/BCAR3 signaling complex to mediate antiestrogen resistance. Testing this model relies on reciprocal analysis of dominant-negative and constitutively active forms of the various signaling molecules in this pathway. As such, bulk of our efforts during the first year have focused on generating the genetic and cellular tools described in the report in detail, allowing us to perform rigorous functional studies on the antiestrogen resistance in breast cancer cells during the upcoming year.

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Table of Contents

Annual Report for W81XWH-04-1-0523

TITLE: CAS SIGNALING IN BREAST CANCER

1. Introduction

Antiestrogens, especially tamoxifen, have proven to be effective in the treatment of hormone-responsive breast cancer. In metastatic breast cancer, antiestrogens lead to a response in nearly one half of patients with estrogen receptor (ER)-positive primary tumor (1). Resistance to antiestrogens, however, is a serious clinical problem. About 40% of ER-positive tumors fail to respond to antiestrogen therapy, and most, if not all, breast tumor patients that initially respond to antiestrogens will eventually develop resistance (2). Furthermore, there are currently no biomarkers that reliably predict tamoxifen responsiveness in patients with ER-positive tumors. A better understanding of the mechanisms of antiestrogen resistance is therefore urgently needed.

A recent mutagenesis approach has identified three independent loci associated with antiestrogen resistance (3), and the target genes of two of the loci, BCAR1 and BCAR3, have been characterized. Interestingly, sequence analysis of BCAR1 demonstrated it to code for the docking protein p130Cas (Cas), which we and others have previously identified to be a key molecule in intracellular signaling pathways (4). Subsequent studies demonstrated that enhanced activation of Cas signaling can induce antiestrogen resistance, at least in cell culture conditions (5). Recent studies have demonstrated that Cas is likely to have a relevant role also in clinical breast cancer; studies on breast cancer samples have shown that high levels of Cas expression correlate with poor relapse-free and overall survival, and the response to tamoxifen therapy in patients with recurrent disease was found to be reduced in patients with primary tumors that expressed high levels of Cas (6).

Our **hypothesis**, which is supported by our preliminary data, is that Cas has an important causal role in the development of antiestrogen resistance. As a corollary, understanding of the pathways that Cas activates may identify key regulators of antiestrogen resistance and novel clinical targets for breast cancer treatment, and measurements of Cas signaling levels may provide useful prognostic information for breast cancer patients. Our **objective** is to test our hypothesis, and to identify and characterize the signaling pathways that mediate Cas-induced antiestrogen resistance. Our additional objective is to develop novel tools to be used as prognostic reagents for ER-positive patients with intrinsic resistance to tamoxifen.

2. Body of the Report

In order to meet the objectives outlined above, two specific aims were set forth in our grant application. **In the first aim**, our goal is to identify and characterize the signaling pathways that mediate Cas-dependent antiestrogen resistance. Our hypothesis is that the interaction of Cas with two signaling molecules, Crk and BCAR3, is required for Cas-dependent antiestrogen resistance. We further hypothesize that the Rac-JNK pathway forms a common pathway downstream of the Cas/Crk/BCAR3 signaling complex to mediate antiestrogen resistance; this working model will be tested in Aim 1 during the first two years of the grant funding. If this model proves to be *in*correct, we will utilize a novel function-based screening method to identify Cas-interacting proteins that mediate antiestrogen resistance. Depending on the nature of the interacting molecules, further experiments will be planned to dissect their roles in antiestrogen resistance. **In the second aim**, to be accomplished during the latter part of the grant period, our goal is to identify the tyrosine residues in Cas that become phosphorylated in breast cancer cells. Cas activates signaling pathways by binding to Src homology 2 (SH2)-domain containing

signaling molecules, such as Crk, in a tyrosine phosphorylation-dependent manner. Further, our preliminary studies indicate that hyperphosphorylation of Cas correlates with antiestrogen resistance. Thus, we will employ two types of mass spectrometers in a multi-tiered strategy to systematically map the tyrosine residues in Cas that become phosphorylated in breast cancer cells *in vivo*.

During the first year of funding, we have focused our efforts on the first aim of the original application, as proposed. **The task** that is to be accomplished as part of this aim is as follows:

Task 1. Test our working model that the Rac-JNK pathway forms a common pathway downstream of the Cas/Crk/BCAR3 complex to mediate antiestrogen resistance (months 1-18).
a. Generate mammalian expression constructs of activated and dominant-negative forms of BCAR3; we already have constructs for Cas, Crk, Rac and the JNK pathway.
b. Test whether expression of an activated form of Crk and activated form of BCAR3 will rescue the CasΔSD and CasΔCT-phenotypes in antiestrogen resistance, respectively. In these studies, stable MCF-7 cell lines expressing the corresponding constructs will be generated, and cell proliferation in the presence of tamoxifen will be studied.
c. Test whether dominant-negative forms of Crk and BCAR3 block Cas-induced antiestrogen resistance. These studies will be performed as above.
d. Determine whether a dominant-negative form of Rac will block Cas-, Crk- and BCAR3-induced antiestrogen resistance. Also determine whether an activated form of Rac will rescue the defect in and inhibition of antiestrogen resistance by CasΔSD and CasΔCT, as well as by dominant-negative forms of Crk and BCAR3. These studies will be performed as above.
e. Determine what is the role of the JNK-pathway downstream of Rac in Cas-mediated antiestrogen resistance. The effect of overexpression of activated and dominant-negative JNK on Cas-,Crk-, BCAR3- and Rac-induced antiestrogen resistance will be studied.

As outlined in Task 1, our experimental strategy for testing our working model that the Rac-JNK pathway forms a common pathway downstream of the Cas/Crk/BCAR3 signaling complex to mediate antiestrogen resistance relies on reciprocal testing of dominant-negative and constitutively active forms of the various signaling molecules in this pathway. As such, bulk of our efforts during the first year of our funding have focused on generating the genetic and cellular tools described above to be able to perform rigorous functional studies on the antiestrogen resistance in breast cancer cells during the upcoming six months. With the recent generation of the DNA constructs for activated and dominant-negative forms of BCAR3, we now have all the necessary DNA tools available that are needed for these studies. Importantly, we have been able to design a point mutant construct of BCAR3 that specifically disrupts binding of BCAR3 to Cas, as opposed to other proteins utilizing the same protein-protein interaction domain within the BCAR/SHEP proteins. Our recent collaborative paper with Dr. Pasquale's laboratory reporting this finding is attached in the Appendix (7). [Other constructs to be used in these studies have been described in our previous studies in refs (8-10)].

As a second step, we are currently generating MCF-7 cell lines expressing physiological or pathophysiological levels of the various constructs described in Task 1. To achieve this objective, we are using DNA constructs generated in a retrovirus background, which allows us to select either clones (to allow selection of a homogenous set of cells) or pools (to eliminate

clonal variation) of MCF-7 cells expressing the desired levels of the exogenously delivered protein(s). We are in the process of selecting several MCF-7 cell lines at the moment. First, we are selecting MCF-7 cell lines overexpressing either Crk or BCAR3 in the CasΔSD or CasΔCT-expressing background, respectively. With these to-be-generated cell lines, we will be able to assess the relative significance of Crk and BCAR3 signaling pathways in Cas-mediated antiestrogen resistance. That is, we will be able to assess as to whether Crk and/or BCAR3 are *sufficient* components in antiestrogen resistance pathways downstream of certain Cas mutants. Second, we are generating cell lines expressing dominant-negative forms of either Crk or BCAR3 in the Cas-overexpressing MCF-7 background. These studies will help us to assess whether Crk and/or BCAR3 are *necessary* components in Cas-mediated antiestrogen resistance pathways. Third, we are generating breast cancer cell lines that overexpress wild-type and activated forms of Crk and BCAR3. Analysis of these cell lines will help us to assess whether Crk and BCAR3 are *necessary* for antiestrogen resistance in breast cancer cell lines *in vitro*. Fourth, cell lines expressing either Cas, Crk, or BCAR3 will be subjected to retroviral infection with a construct expressing a dominant-negative form of Rac. This line of investigation will allow us to assess the putative *necessary* role of the small GTPase Rac in antiestrogen resistance mediated by Cas, Crk and/or BCAR3. Finally, we are generating breast cancer cell lines that express the activated form of Rac in a CasΔSD or CasΔCT-expressing MCF-7 background. This study will allow us to assess whether Rac is a *sufficient* component in Cas-mediated antiestrogen signaling pathways.

In conclusion, we are well on schedule in accomplishing our proposed studies within the Task 1 during the first 18 months of our funding period. The results outlined above, as well as some additional results from related studies, will be presented in the Era of Hope meeting in May.

3. Key Research Accomplishments

1. Generation of all of the DNA constructs needed to accomplish studies outlined in Aim 1 of the original application.

2. By virtue of generating the requisite DNA constructs (see above), we are well on schedule to generate the proposed breast cancer cell lines in Task 1 in order to rigorously examine the working model of this grant application; that is, that the Rac-JNK pathway forms a common pathway downstream of the Cas/Crk/BCAR3 signaling complex to mediate antiestrogen resistance in breast cancer.

4. Reportable Outcomes

1. Generation of molecularly-defined key DNA constructs and breast cancer cell lines to examine antiestrogen resistance *in vitro*.

5. Conclusions

Our preliminary data presented in the original grant application supports the role of the docking protein Cas in antiestrogen resistance. During the first year of funding, we have accomplished the bulk of the goals outlined in Aim 1; in these studies, the intracellular signaling pathways downstream of Cas mediating antiestrogen resistance will be interrogated in detail at the molecular level. Immediate next studies will take advantage of the newly generated tools described above, in studies also outlined above. No changes have been made, nor proposed, to the technical design to accomplish the goals of the original application.

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7. Appendix

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SHEP1 Function in Cell Migration Is Impaired by a Single Amino Acid Mutation That Disrupts Association with the Scaffolding Protein Cas but Not with Ras GTPases*

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SHEP1 is a signaling protein that contains a guanine nucleotide exchange factor-like domain, which binds Ras family GTPases and also forms a stable complex with the scaffolding protein Crk-associated substrate (Cas). SHEP1 and Cas have several common functions, such as increasing c-Jun N-terminal kinase activity, promoting T cell activation, and regulating the actin cytoskeleton. However, it is unclear whether a physical association between SHEP1 and Cas is required for these activities. We reported previously that SHEP1 is tyrosine-phosphorylated downstream of the EphB2 receptor; in this study, we further demonstrate that activated EphB2 inhibits SHEP1 association with Cas. To investigate whether phosphorylation negatively regulates the SHEP1-Cas complex, we have identified by mass spectrometry several SHEP1 tyrosine phosphorylation sites downstream of EphB2; of particular interest among them is tyrosine 635 in the Cas association/exchange factor domain. Mutation of this tyrosine to glutamic acid, but not to phenylalanine, disrupts Cas binding to SHEP1 without inhibiting Ras GTPase binding. The glutamic acid mutation also makes SHEP1 unable to promote Cas-Crk association, membrane ruffling, and cell migration toward epidermal growth factor (EGF), implying that these activities of SHEP1 depend upon a physical interaction with Cas. Association with Cas also seems to be necessary for EGF-induced SHEP1 tyrosine phosphorylation, which is mediated by a Src family kinase. It is noteworthy that EGF stimulation does not cause dissociation of SHEP1 from Cas. These data show that SHEP1 regulates membrane ruffling and cell migration and that binding to Cas is probably critical for these functions. Furthermore, the SHEP1-Cas complex may have different roles downstream of EphB2 and the EGF receptor.

Cell migration is crucial during embryonic development to sort and position cells to their appropriate location within a tissue (1). It also plays a critical role in tissue repair, immune surveillance, inflammation, and cancer invasion and metastasis. The actin cytoskeleton is constantly undergoing reorganization in moving cells, concomitant with the formation and breakdown of cell substrate adhesion sites at the leading and trailing edges of the cell (1, 2). At these adhesion sites, integrins mediate cell attachment to extracellular matrix components. Receptor tyrosine kinases, on the other hand, control the direction of cell movement in response to attractive and repellent cues.

Crk-associated substrate (Cas),[1] a docking protein found at cell substrate adhesion sites, plays an important role in cell migration downstream of both integrins and receptor tyrosine kinases (3, 4). Cas serves as a scaffold for multiple proteins through its various protein interaction modules, including an amino-terminal SH3 domain and multiple binding sites for SH2 and SH3 domain-containing proteins. SH2 domain-containing proteins bind many tyrosine motifs in Cas that become phosphorylated upon integrin-mediated cell attachment and receptor tyrosine kinase activation. Many binding partners for Cas have been identified thus far, including nonreceptor tyrosine kinases such as focal adhesion kinase and Src, cytoplasmic protein tyrosine phosphatases, and several adaptor proteins. The ability to form complexes with such a variety of signaling molecules enables Cas to coordinate diverse signals that influence cell behavior.

A well characterized Cas signaling interaction involves recruitment of multiple copies of the Crk adaptor to tyrosine-phosphorylated Cas, resulting in activation of downstream signaling pathways. The Cas-Crk complex promotes cell substrate adhesion through the Crk-associated exchange factor C3G, which activates the small GTPase Rap1 (5–9). The Cas-Crk complex also promotes membrane ruffling and cell migration through DOCK180, a novel Crk-associated exchange factor that activates the Rac GTPase (10–13).

A new family of Cas-binding proteins includes SHEP1 (also known as Chat and Nsp3), BCAR3 (also known as AND-34, SHEP2, and Nsp2), and Nsp1 (14–18). The proteins of this family, which we refer to as the SHEP family, have a distinc-

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[1] The abbreviations used are: Cas, Crk-associated substrate; JNK, c-Jun NH_2-terminal kinase; SH, Src homology; CA, Cas association; GEF, guanine nucleotide exchange factor; EGF, epidermal growth factor; GST, glutathione *S*-transferase; FBS, fetal bovine serum; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight; DMEM, Dulbecco's modified Eagle's medium; DAPI, 4,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein; CasΔSD, Cas lacking the substrate domain.

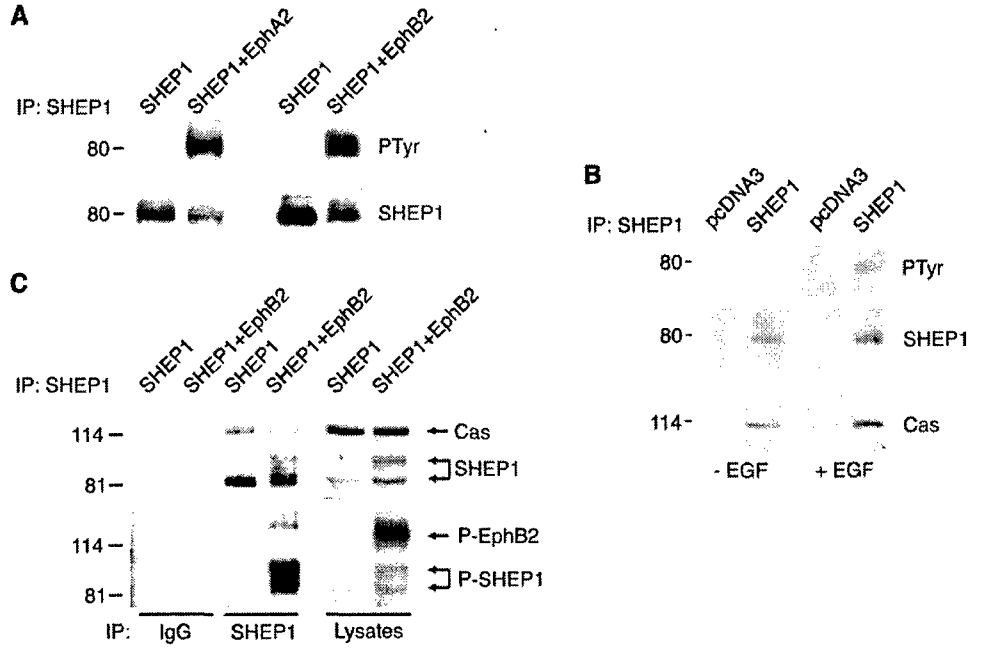

FIG. 1. **Eph receptors and the EGF receptor both cause SHEP1 tyrosine phosphorylation but have different effects on SHEP1-Cas association.** *A*, the EphA2 and EphB2 receptors, which are activated when overexpressed in 293 cells, induce tyrosine phosphorylation of SHEP1. SHEP1 was immunoprecipitated (*IP*) from cells transfected with SHEP1 alone or together with EphA2 or EphB2. The immunoprecipitates were probed with anti-phosphotyrosine (*PTyr*) antibodies and reprobed with anti-SHEP1 antibodies. *B*, EGF stimulation of COS cells induces tyrosine phosphorylation of SHEP1 without inhibiting SHEP1-Cas association. SHEP1 was immunoprecipitated from COS cells transfected with SHEP1 and treated with EGF for 5 min or left untreated. The immunoprecipitates were probed with anti-phosphotyrosine antibodies and reprobed with anti-SHEP1 and anti-Cas antibodies. *C*, activated EphB2 inhibits the association of SHEP1 with Cas. *Top*, immunoprecipitates with anti-SHEP1 antibodies or non-immune rabbit IgG were probed with anti-Cas (*upper half*) and anti-SHEP1 (*lower half*) antibodies. *Bottom*, reprobing with anti-phosphotyrosine antibodies verified tyrosine phosphorylation of SHEP1 (*P-SHEP1*) and EphB2 (*P-EphB2*) in EphB2-transfected 293 cells. SHEP1 appears as a doublet in EphB2-transfected cells probably because of phosphorylation (14, 18).

tive domain structure. They contain an amino-terminal SH2 domain followed by a proline/serine-rich region and a carboxyl-terminal domain with homology to the nucleotide exchange factor domain of Cdc25 (14). Although the exchange factor-like domain of the SHEP proteins binds several Ras family GTPases, it remains unclear whether it has the ability to promote nucleotide exchange (14, 19, 20). This region of the SHEP proteins also mediates the association with Cas (16–18). This interaction does not require tyrosine phosphorylation, and Cas and SHEP proteins can be readily co-immunoprecipitated from tissues and cultured cells (16–18). The SHEP-Cas complex can bind to activated receptor tyrosine kinases through the SHEP SH2 domain and to integrins through Cas-mediated interactions. Thus, this complex has the characteristics of an integrator of signals from different classes of cell surface receptors.

Increasing evidence suggests that SHEP proteins and Cas have a shared signaling function affecting cell proliferation and motility (15, 18, 21–24). However, it is unclear whether a physical association between SHEP proteins and Cas is required to trigger these cellular responses. Recent experiments with mutated proteins show that the SHEP family protein AND-34 causes relocalization of Cas to membrane ruffles and increased Src activity even in the absence of a direct interaction with Cas (24). Other findings support the importance of the SHEP1-Cas association for promoting cell polarization and other changes in cell morphology, increased c-Jun NH_2-terminal kinase (JNK) activity, and T cell activation (22–24). There are ambiguities in the interpretation of these experiments, however, because the structurally disruptive mutations that were used to evaluate the role of SHEP-Cas interaction probably affect multiple functions of either Cas or the SHEP proteins. For example, truncated SHEP proteins lacking the Cas-association domain also lack the exchange factor-like domain, making it difficult to distinguish the importance of the interaction with Cas *versus* the interaction with Ras GTPases.

Herein, we identify tyrosine 635 in the Cas association-guanine nucleotide exchange factor (CA-GEF) domain of SHEP1 as a critical and selective site for SHEP1-Cas interaction because mutation of this single residue to glutamic acid (Y635E) disrupts the ability of SHEP1 to bind Cas without inhibiting the binding of Ras GTPases. It is interesting that the selectivity of the Y635E mutation indicates that Cas and Ras proteins have distinct modes of binding to the CA-GEF domain of SHEP1. Furthermore, the effects of the SHEP1 Y635E mutation suggest that the association of SHEP1 with Cas is required to promote epidermal growth factor (EGF)-dependent SHEP1 phosphorylation by Src, membrane ruffling, and cell migration toward EGF.

EXPERIMENTAL PROCEDURES

Plasmids—The pcDNA3-SHEP1 vector contains nucleotides 33–2165 of the short isoform of mouse SHEP1 (also known as Chat) (GenBank™ accession number AB030442). This isoform was used because it is more widely expressed outside the hematopoietic system (18). The pcDNA3-SHEP1 vector was used as a template to obtain the SHEP1 Y635F and Y635E mutants by using overlapping PCR amplification with the forward primer 5′-GGGCCTCTTCCACACCAAC-3′ (the mutated nucleotide introducing the Y635F mutation is underlined) and the reverse primer (5′-GTTGGTGTGCTCGAGGCCCCC-3′) (the mutated nucleotides introducing the Y635E mutation are underlined). The myr-SHEP1 and myrSHEP Y635E constructs were obtained by subcloning the corresponding SHEP1 cDNAs into a pcDNA3 vector encoding the Src myristoylation signal and some linker amino acids (MGSSKSKPK-DPSQREFCRYPHSWRPLEGIDKLGTELGSA) fused to the amino terminus of SHEP1. The pEGFP-Rap2 plasmid encodes nucleotides 252–818 of mouse Rap2A (GenBank™ accession number NM_029519) cloned into the pEGFP-C2 vector (BD Biosciences Clontech). The pSSRα-CasΔ133 plasmid encodes rat Cas lacking the 133 carboxyl-terminal amino acids; pcDNA3-kinase inactive Src encodes chicken Src with a K295M mutation, pcDNA3-EphA2 encodes full-length human

EphA2 (25), and pcDNA3-EphB2 encodes full-length chicken EphB2 (26). pGEX plasmids were engineered encoding the following fusion proteins: GST-Cas 258 (Cas amino acids 711–968), GST-Cas 204 (Cas amino acids 765–968), GST-Cas 133 (Cas amino acids 836–968), and GST-SHEP1 CA-GEF (amino acids 362–702 of mouse SHEP1). Other plasmids have been described previously: pSSRα-Cas (28), pSSRα-CasΔS.D (29), pCAGGS-myc-Crk (30), and pcDNA3-myc-R-Ras (54). pEGFP-N vectors encoding enhanced green fluorescent protein were from BD Biosciences Clontech.

Antibodies—Anti-EphB2 antibodies were obtained using as the antigen a GST fusion protein comprising amino acids 897–995 of chicken EphB2, as described previously (31). Anti-SHEP1 antibodies were made using a GST fusion protein of the SHEP1 SH2 domain, as described previously (14), or a peptide corresponding to the 13 carboxyl-terminal amino acids of SHEP1 cross-linked to bovine serum albumin. The SHEP1 peptide antibodies were purified on affinity columns containing either the same SHEP1 peptide used for immunization or GST-SHEP1 CA-GEF. Anti-Cas, Crk, and Rap2 monoclonal antibodies and anti-phosphotyrosine monoclonal antibodies conjugated to horseradish peroxidase were from BD Transduction Laboratories. CasΔ133, however, was detected with anti-Cas antibody N-17 (Santa Cruz Biotechnology Inc.) Anti-Src monoclonal antibody was from Upstate, anti-myc 9E10 was from Sigma, and secondary anti-mouse and anti-rabbit IgG peroxidase-conjugated antibodies were from Amersham Biosciences.

Cell Culture and Transfections—Human embryonal kidney 293T cells, COS cells, and NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. Transient transfections of 293T and COS cells were carried out using Superfect Transfection Reagent (Qiagen Inc.). Cells were harvested 48 to 72 h after transfection. NIH3T3 cells were transfected using LipofectAMINE Plus reagent (Invitrogen). For EGF stimulation experiments, cells were serum-starved in DMEM with 0.5% FBS for 16 h before stimulation with 100 ng/ml EGF (Sigma). The Src kinase inhibitor PP2 (Calbiochem) was dissolved at 10 mM in Me_2SO and added 30 min before EGF treatment at a final concentration of 10 μM. Me_2SO alone was used as a control.

Immunoprecipitation and Immunoblotting—Transiently transfected cells were lysed in RIPA buffer containing 10 mM NaF, 1 mM sodium pervanadate, and protease inhibitors (Fig. 1A and Fig. 6) or Brij buffer (1% Brij 97 in phosphate-buffered saline) containing 5 mM EDTA, 10 mM NaF, 1 mM sodium pervanadate, and protease inhibitors. For immunoprecipitations, cell lysates were incubated with 7–15 μg of anti-SHEP1 antibody, 5 μg of anti-Cas antibody, or 4 μg of anti-Crk antibody immobilized on GammaBind Plus Sepharose beads (Amersham Biosciences). Immunoprecipitates were separated by SDS-PAGE and probed by immunoblotting. Detection of horseradish peroxidase-conjugated secondary antibodies was performed with enhanced chemiluminescence detection systems from Amersham Biosciences or Pierce. After an initial immunoblot, some filters were stripped and then reprobed with different antibodies.

GST Pull-down Experiments—Hippocampal lysates were prepared using a modified RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.2% sodium deoxycholate, 0.02% SDS, 1 mM EDTA, 5 mM dithiothreitol, and 20 mM Tris-HCl pH 8.0) containing 1 mM sodium pervanadate and protease inhibitors. GST-SHEP1 CA-GEF immobilized on glutathione beads was incubated with hippocampal lysate for 1 h. Pull-down experiments with GST-Cas fusion proteins were performed for 45 min with 293T cells transiently transfected with myrSHEP1 and lysed in Brij buffer (see previous section).

MALDI-TOF Mass Spectrometry—293T cells transiently transfected with SHEP1 and EphB2 or EphB2 alone were lysed in RIPA buffer containing protease inhibitors, 1 mM sodium orthovanadate, 10 mM NaF, and 5 mM EDTA. Lysates were incubated with 100 μg of anti-SHEP1 antibodies bound to 30 μl of GammaBind Plus Sepharose for 2 h at 4 °C. The samples were washed three times in the above buffer, boiled in 1% SDS, and reprecipitated (32). The samples were then washed three times in the above buffer and three times in 0.1% 1-*O*-octyl-β-D-glucopyranoside (98%) and 100 mM NH_4HCO_3, pH 7.5, containing 1 mM sodium orthovanadate. Endoproteinase Glu-C/trypsin proteolytic digestions were performed with sequence grade proteases in 100 μl of100 mM NH_4HCO_3, pH 8.0, at 37 °C overnight with endoproteinase Glu-C (50 ng/μl) first, followed by a overnight tryptic digestion at 37 °C (50 ng/μl). Endoproteinases were inhibited with phenylmethylsulfonyl fluoride (100 μg/ml). The digested samples were centrifuged, and the supernatants were removed. The sedimented beads were washed twice with 200 L of 100 mM NH_4HCO_3, pH 8.0, containing phenylmethylsulfonyl fluoride and the supernatant and washes were combined with 20 μl of anti-phosphotyrosine antibodies conjugated to agarose (33) for overnight incubation at 4 °C. The beads were washed four times with 100 mM NH_4HCO_3, pH 8, and then packed into a 10-μl Geloader Tip (Eppendorf). The beads in the tip were washed three times with 60 μl of 100 mM NH_4HCO_3, pH 8. The phosphopeptides were eluted in 20-μl aliquots of 100 mM phosphate, pH 2.5, directly into a zip tip packed with C_{18}, which had been equilibrated by washing 3 times with 20 μl of 75% acetonitrile and 0.1% TFA followed three times with 20 μl of 0.1% TFA. After cycling each acid eluate through the zip tip five times, the tip was washed five times with 20 μl of 0.1% TFA. For samples submitted for MALDI analysis, phosphopeptides were eluted from the zip tip in 3 μl of 10 mg/ml α-cyano-4-hydroxcinnamic acid in 3 μl of 75% acetonitrile and 0.1% TFA directly onto a target.

Mass spectra were collected on a Voyager matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometer equipped with a nitrogen laser, delayed extraction, and a reflector. Spectra were externally calibrated with angiotensin I (MH^+ = 1296.69), and adrenocorticotropic hormone fragments (1–17, MH^+ = 2093.09; 18–39, MH^+ = 2465.20; 7–38, MH^+ = 3657.93). For interpretation of the mass spectra, a list of predicted molecular masses was generated by theoretical cleavage with specific endoproteinases using the MS-Digest program (prospector.ucsf.edu). Each peptide was assumed to contain at least one phosphate group. The masses of the peaks recorded in the mass spectra were matched to the calculated masses with an accuracy of at least 0.15%.

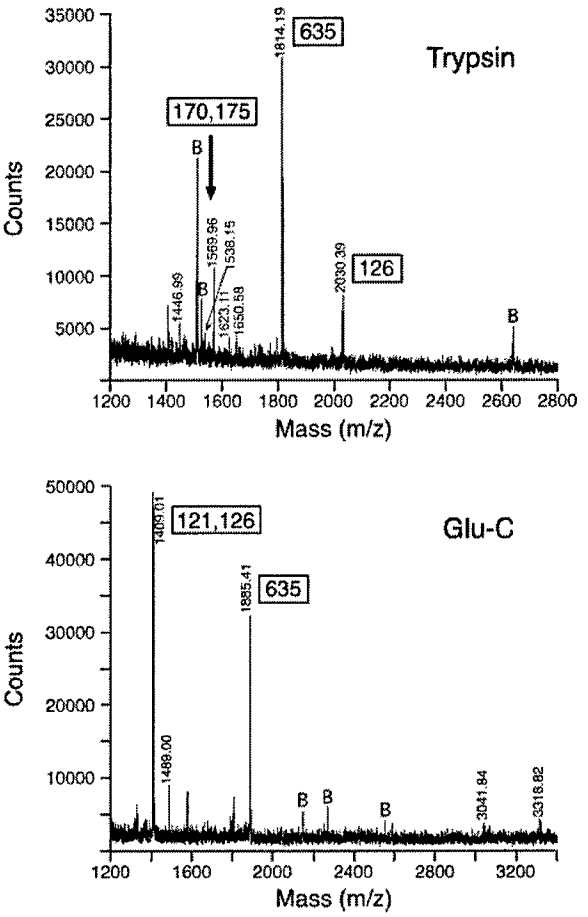

FIG. 2. **Multiple tyrosines of SHEP1 are phosphorylated downstream of EphB2.** MALDI-TOF mass spectra of tyrosine-phosphorylated peptides from SHEP1 immunoprecipitated from 293 cells co-transfected with EphB2 and SHEP1 and digested with trypsin or endoproteinase Glu-C. Tyrosine-phosphorylated peptides were isolated by binding to anti-phosphotyrosine antibodies conjugated to agarose, followed by purification by reversed-phase chromatography. Peaks corresponding to tyrosine-phosphorylated peptides of SHEP1 are denoted by their masses and identified in Table I. The tyrosine residues contained in peptides corresponding to major peaks are also indicated in boxes. *B*, background peaks from cells transfected with EphB2 but not SHEP1. Prominent peaks in the Glu-C and trypsin digests represent different monophosphorylated peptides containing tyrosine 635 in the SHEP1 CA-GEF domain.

TABLE I
Phosphotyrosine peptides from SHEP1 digested with trypsin or endoproteinase Glu-C

Tyrosine residue	Mass		Mono or avg[a]	%Δ[b]	Peptide[c]
	Measured	Calculated			
	Da				
Trypsin					
121	1113.63	1113.47	m	0.014	AGESYTHIR + $1PO_4$[d]
26	1417.87	1417.63	m	0.017	TAAELEAAGDYVK + $1PO_4$
560	1446.99	1448.62	m	0.113	HTEGAILYEKK + $2PO_4$
121	1538.15	1538.77	m	0.041	VVVKAGESYTHIR + $1PO_4$
64	1538.15	1539.64	m	0.097	SHAWYHGRIPR + $2PO_4$
170, 175	1569.96	1569.68	m	0.018	YLEASYGLSQGSSK + $1PO_4$
95	1623.11	1622.71	m	0.024	DSLTSLGDYVLTCR + $1PO_4$
170, 175	1650.70	1650.58	a	0.007	YLEASYGLSQGSSK + $2PO_4$
635	1814.19	1813.84	m	0.019	TVAHHGGLYHTNAEVK + $1PO_4$
126	2030.39	2029.94	m	0.022	YLFEQESFDHVPALVR + $1PO_4$
Endoproteinase Glu-C					
121, 126	1409.01	1408.63	m	0.027	SYTHIRYLFE + $1PO_4$
121, 126	1489.00	1488.60	m	0.027	SYTHIRYLFE + $2PO_4$
635	1885.41	1884.85	m	0.030	AARTVAHHGGLYHTNAE + $1PO_4$
26, 34	3041.84	3044.24	a	0.079	AAGDYVKFSKEKYILDSSPEKLHKE + $2PO_4$
679	3318.82	3320.37	a	0.047	VFSTEFQMRLLWGSQGANSSQAWRYE + $3PO_4$

[a] m, monoisotopic mass; a, average mass.
[b] Percentage difference between measured mass and calculated mass.
[c] Tyrosine residues are indicated in bold.
[d] PO_4 indicates a phosphate group.

Membrane Ruffling—Transiently transfected NIH3T3 cells were starved overnight in DME with 0.5% FBS starting 24 h after transfection. Twelve hours later, cells were trypsinized with 0.05% trypsin in EDTA (Invitrogen), collected in DME with 0.5% BSA, washed, and kept in suspension for 45 min at 37 °C. The cells were then allowed to attach on fibronectin-coated coverslips for 3 h, fixed with 4% formaldehyde in phosphate-buffered saline, permeabilized in 0.1% TX-100 in phosphate-buffered saline, and stained with Alexa 594 phalloidin (Molecular Probes) and DAPI (Molecular Probes). Co-expressed enhanced green fluorescent protein (EGFP) was used to identify transfected cells. For quantification, cells with ruffles were counted by an investigator blind to the transfected protein.

Cell Migration—Transiently transfected COS cells were starved in DME with 0.5% FBS for 16 h. Cells (100,000 cells/well) were then seeded on Transwell filters (Corning Inc.) that had been coated on both sides with 10 μg/ml fibronectin and blocked with 1% BSA. Cells were allowed to migrate toward 20 ng/ml EGF in DMEM and 0.5% FBS. After migration, cells on the upper side of the filters were removed, and the filters were fixed in 4% formaldehyde, permeabilized in 0.5% TX-100 in phosphate-buffered saline, stained with DAPI, and mounted on glass slides. Transfected cells (positive for EGFP and DAPI) that had migrated to the bottom side of the filters were counted under a fluorescent microscope and expressed as the number of cells in 10 microscope fields (60× magnification). Untransfected cells (positive only for DAPI) were also counted separately as a control (not shown). Transfection efficiencies were determined from separate aliquots of transfected cells that were plated on glass coverslips. Lysates from the transfected cells were also probed by immunoblotting to verify expression of the transfected proteins (not shown).

RESULTS

Eph Receptors and the EGF Receptor Both Enhance SHEP1 Tyrosine Phosphorylation but Have Different Effects on the Association of SHEP1 with Cas—We reported previously that SHEP1 is phosphorylated on tyrosine residues downstream of EphB2 (14). Immunoprecipitation of SHEP1 from extracts of 293 human embryonal kidney cells expressing activated EphA2, followed by immunoblotting with anti-phosphotyrosine antibodies, showed that SHEP1 is similarly phosphorylated downstream of EphA2 (Fig. 1*A*). Hence, SHEP1 is a target of both EphA and EphB receptors. Other receptor tyrosine kinases, such as the EGF and NGF receptors, have been reported to enhance SHEP1 (Chat) phosphorylation on serine/threonine but not tyrosine residues in PC-12 cells (18). In COS cells, however, we readily detected SHEP1 tyrosine phosphorylation after EGF receptor activation (Fig. 1*B*), as discussed by others (18). Thus, activation of different families of receptor tyrosine kinases can induce SHEP1 tyrosine phosphorylation.

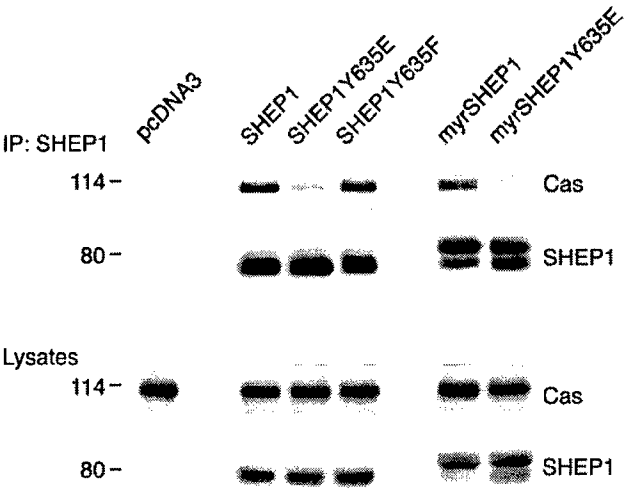

FIG. 3. **Mutation of SHEP1 tyrosine 635 to glutamic acid inhibits Cas binding.** 293 cells transfected with the indicated constructs were used for immunoprecipitation (*IP*) with anti-SHEP1 antibodies and probed with anti-Cas antibodies to detect association of endogenous Cas.

Because tyrosine phosphorylation is a well known mechanism for regulating protein interactions, we next examined whether the EGF receptor and EphB2 have an effect on the association of SHEP1 with Cas. We detected less Cas bound to SHEP1 in cells expressing activated EphB2 (Fig. 1*C*). In contrast, EGF receptor activation did not inhibit the interaction between SHEP1 and Cas (Fig. 1*B*).

Activated EphB2 Causes Phosphorylation of Tyrosine 635 in the SHEP1 CA-GEF Domain—To identify the tyrosine phosphorylation sites of SHEP1 in EphB2-tranfected cells, which may disrupt the association of SHEP1 with Cas, we used a MALDI-TOF mass spectrometry approach. SHEP1 was isolated by immunoprecipitation from 293 cells transfected with both SHEP1 and EphB2 and digested with endoproteinase Glu-C or trypsin. Tyrosine-phosphorylated peptide fragments from the immunoprecipitates were isolated with anti-phosphotyrosine antibodies conjugated to agarose, purified by reversed-

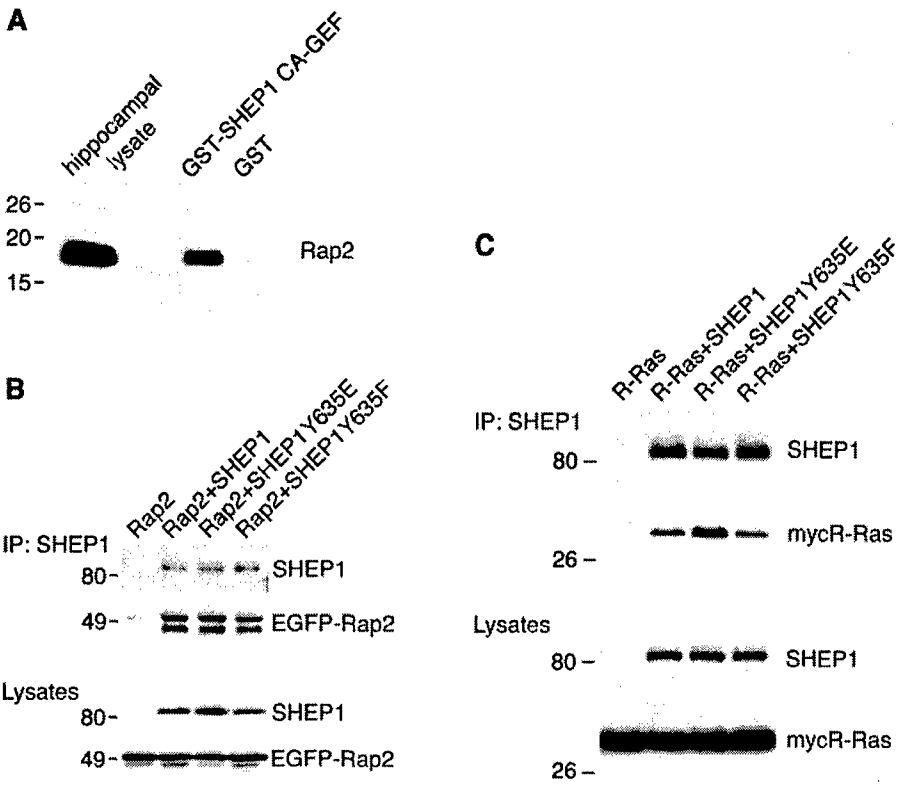

FIG. 4. **Mutation of SHEP1 tyrosine 635 to glutamic acid does not affect Rap2 and R-Ras binding.** *A*, Rap2 binds to the CA-GEF domain of SHEP1. A GST fusion protein of the SHEP1 CA-GEF domain, or GST as a control, were used to pull down Rap2 from hippocampal lysates, where endogenous Rap2 is expressed at high levels. *B* and *C*, 293 cells transfected with the indicated SHEP1 constructs, and EGFP-Rap2 or myc-R-Ras was used for immunoprecipitations (*IP*) with SHEP1 antibodies. The immunoprecipitates were probed by immunoblotting with anti-Rap2, anti-myc, and anti-SHEP1 antibodies.

phase chromatography, and analyzed by mass spectrometry. Prominent peaks in the mass spectra from both Glu-C and trypsin digested SHEP1 correspond to a number of SHEP1 phosphorylated peptides containing tyrosine residues (Fig. 2, Table I). A major peak in both spectra corresponds to a peptide containing tyrosine 635 in the CA-GEF domain of SHEP1, which is the region that binds Cas as well as Ras family proteins. This led us to focus on tyrosine 635 of SHEP1 as a possible site of regulation by phosphorylation.

Mutation of SHEP1 Tyrosine 635 to Glutamic Acid Inhibits Cas Binding without Affecting the Binding of Ras GTPases—To evaluate the importance of tyrosine 635 in the association of SHEP1 with Cas, we used site-directed mutagenesis to replace this tyrosine with glutamic acid (Y635E mutation), which introduces a negative charge like that of a phosphate group (34). We also generated a SHEP1 Y635F mutant, in which the phenylalanine cannot be phosphorylated. Co-immunoprecipitation experiments revealed that the glutamic acid mutation disrupts the association of SHEP1 with endogenous Cas, whereas the phenylalanine mutation does not (Fig. 3).

In addition to Cas, the CA-GEF domain of SHEP1 is known to bind the small GTPases R-Ras and Rap1 (14). As shown in Fig. 4*A*, the SHEP1 CA-GEF domain also binds another GTPase, Rap2. It is interesting that in co-immunoprecipitation experiments, SHEP1 Y635E binds Rap2 similarly to wild-type SHEP1 and the Y635F mutant (Fig. 4*B*). The binding of R-Ras is also not inhibited by the Y635E and Y635F mutations (Fig. 4*C*). Thus, mutation of tyrosine 635 does not disrupt the overall three-dimensional structure of the SHEP1 CA-GEF domain because the mutant SHEP1 retains the ability to bind Ras family GTPases. The Y635E mutation represents an important tool for elucidating the function of the SHEP1-Cas complex because it allows selective inhibition of Cas binding without disrupting binding of Ras proteins.

Mutation of SHEP1 Tyrosine 635 to Glutamic Acid Inhibits SHEP1 Tyrosine Phosphorylation Downstream of the EGF Receptor but Not of EphB2—The remarkable effect of the Y635E mutation suggests that phosphorylation of SHEP1 at this site inhibits association with Cas. If this were the case, tyrosine 635 would not be expected to be phosphorylated in EGF-stimulated cells where SHEP1 and Cas remain in complex. We indeed found that after EGF stimulation, the SHEP1 Y635F mutant was phosphorylated at levels similar to those of wild-type SHEP1 (Fig. 5*A*). This result, which was also obtained by blotting with the anti-phosphotyrosine antibody used for the mass spectrometry experiments (data not shown), suggests that tyrosine 635 is not phosphorylated downstream of the EGF receptor or that inhibiting phosphorylation at this site may increase phosphorylation of other tyrosines. In contrast, EGF stimulation did not detectably increase tyrosine phosphorylation of SHEP1 Y635E (Fig. 5*B*). Because the Y635E and 635F mutants lack the same tyrosine, but the Y635E mutant in addition does not bind Cas, this result suggests that binding to Cas is important for SHEP1 phosphorylation on other tyrosine residues downstream of the EGF receptor. Because Src binds to Cas and is activated upon EGF-stimulation, we examined the involvement of Src in EGF-dependent SHEP1 phosphorylation. Treatment with the Src family kinase inhibitor PP2 decreased SHEP1 phosphorylation to undetectable levels (Fig. 5*C*), indicating that a Src family kinase rather than the activated EGF receptor phosphorylates SHEP1. These results suggest that Cas serves to couple SHEP1 to Src. In contrast, we found that both SHEP1 Y635F and Y635E mutants are substantially phosphorylated downstream of EphB2, although less efficiently than wild-type SHEP1 (Fig. 5*D*). This suggests a different mechanism for SHEP1 tyrosine phosphorylation downstream of EphB2, which does not require Cas binding.

Membrane-Targeting of SHEP1 Enhances Cas Phosphorylation by Src and Coupling to Crk—Binding to phosphorylated motifs of activated Eph and EGF receptors presumably localizes SHEP1 near the plasma membrane (18) and also near Src, which is recruited by these receptors (35, 36). To evaluate the consequences of SHEP1 membrane-targeting independently of receptor tyrosine kinase activation, we fused the Src myristoyl-

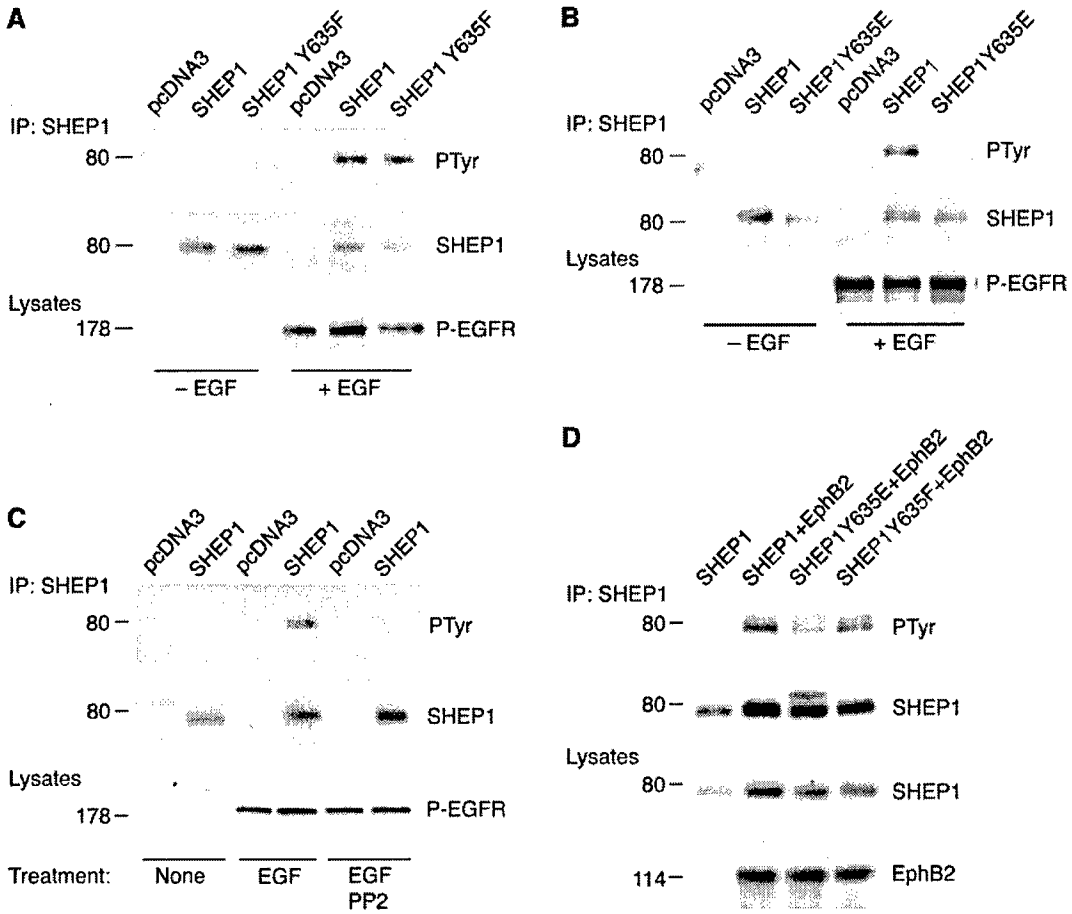

FIG. 5. **SHEP1 phosphorylation downstream of the EGF receptor requires Cas association and Src activity.** *A* and *B*, mutation of tyrosine 635 of SHEP1 to glutamic acid but not phenylalanine inhibits SHEP1 phosphorylation downstream of the EGF receptor. SHEP1 was immunoprecipitated (*IP*) from COS cells transfected with the indicated constructs and stimulated with EGF for 5 min (*A*) or 3 min (*B*) or left untreated. *C*, the Src kinase inhibitor PP2 inhibits SHEP1 tyrosine phosphorylation in EGF-stimulated COS cells. Cells were pretreated with PP2 and stimulated with EGF for 5 min before immunoprecipitating SHEP1. *D*, mutation of tyrosine 635 to glutamic acid does not abolish SHEP1 phosphorylation downstream of EphB2 in COS cells. In all panels, the immunoprecipitates were probed with anti-phosphotyrosine (*PTyr*) antibodies and reprobed with anti-SHEP1 antibodies. Lysates were probed with anti-phosphotyrosine antibodies to detect phosphorylation of the EGF receptor (*P-EGFR*).

ation signal to the amino terminus of SHEP1. When transfected into 293T cells, this membrane-targeted myrSHEP1 became tyrosine phosphorylated and also enhanced Cas phosphorylation (Fig. 6, *A–C*). The association of SHEP1 with Cas is probably important for the increased Cas phosphorylation because a myrSHEP1 Y635E mutant, which does not bind Cas, had only a weak effect on Cas phosphorylation (Fig. 6*B*). Furthermore, co-transfection of myrSHEP1 with kinase inactive Src, which acts as a dominant-negative mutant for Src family kinases, inhibited the increase in Cas tyrosine phosphorylation (Fig. 6*C*). These results implicate Src family kinases, which are known to phosphorylate Cas under a variety of conditions (3, 4), in Cas phosphorylation induced by myrSHEP1.

Signals triggered by tyrosine phosphorylation of both Cas and SHEP1 may contribute to the functions of the SHEP1-Cas complex. Consistent with the known role of Cas tyrosine phosphorylation in promoting Crk binding (3, 4), co-immunoprecipitation experiments revealed an increased association of Cas with Crk in cells expressing myrSHEP1 (Fig. 7*A*). In contrast, expression of the myrSHEP1 Y635E mutant did not enhance Crk binding to Cas (Fig. 7*B*). Taken together, the results shown in Figs. 6 and 7 suggest that SHEP1 serves to recruit Cas to the plasma membrane, where Cas is phosphorylated by a Src family kinase and initiates Crk-dependent downstream signaling.

SHEP1-Cas Interaction Promotes Membrane Ruffling—Crk recruitment to Cas is known to activate downstream signaling pathways that regulate membrane ruffling and cell motility (37, 38). We used NIH3T3 cells to evaluate the effects of SHEP1 on membrane ruffling because of the uniform shape of these cells and their low levels of spontaneous ruffling. Constructs encoding myrSHEP1, myrSHEP1 Y635E, and pcDNA3 vector control were co-transfected with EGFP to identify transfected cells. As shown in Fig. 8, myrSHEP1 dramatically increased membrane ruffling in cells that were allowed to adhere on a fibronectin substrate for 3 h, whereas myrSHEP1 Y635E did not. About 60% of the cells expressing myrSHEP1 had ruffles *versus* less than 10% of the cells transfected with myrSHEP1 Y635E or pcDNA3 vector control. Ruffles were also present in only 10% or less of the untransfected cells. To examine the mechanism by which the SHEP1-Cas complex promotes ruffling, we co-transfected myrSHEP1 together with a mutant form of Cas lacking the substrate domain (CasΔSD), which is the region that binds Crk. CasΔSD, which has been shown to act as a dominant-negative form of Cas by preventing coupling of Cas and Crk (11), substantially decreased myrSHEP1-mediated membrane ruffling (Fig. 8, *C* and *D*). These results suggest that the association between myrSHEP1 and Cas and Cas downstream signaling are important for the ruffling effects.

SHEP1-Cas Interaction Promotes EGF-dependent Cell Migration—To examine the importance of SHEP1 and its association with Cas in cell migration toward EGF, we transfected COS cells with wild-type or mutated SHEP1 together with Cas.

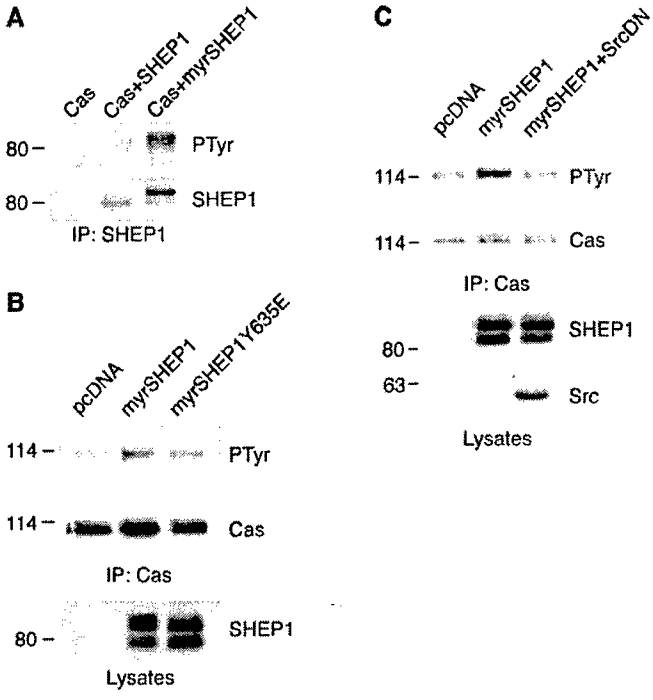

FIG. 6. **Membrane-targeted SHEP1 is tyrosine phosphorylated and increases Cas phosphorylation by a Src kinase.** *A*, myrSHEP1, which is membrane-targeted by a myristoylation signal, is tyrosine phosphorylated. *B*, myrSHEP1 causes increased Cas tyrosine phosphorylation, whereas the myrSHEP1 Y635E mutant does not. 293 cells (*A*) or COS cells (*B*) transfected with the indicated constructs were used for immunoprecipitation (*IP*) with anti-SHEP1 (*A*) or anti-Cas (*B*) antibodies. Immunoprecipitates were probed with anti-phosphotyrosine antibodies (PTyr) and re-probed with the immunoprecipitating antibody. *C*, dominant-negative, kinase-inactive Src (*Src DN*) inhibits Cas phosphorylation in 293 cells expressing myristoylated SHEP1, suggesting that a Src kinase phosphorylates Cas. Cas immunoprecipitates were probed with anti-phosphotyrosine antibodies and reprobed with anti-Cas antibodies.

EGFP was used to identify the transfected cells. In transwell migration assays, SHEP1 co-expressed with Cas significantly enhanced cell migration toward a low concentration of EGF (Fig. 9*A*). The SHEP1 Y635F mutant also enhanced cell migration, whereas the SHEP1 Y635E mutant—which does not bind Cas—did not exhibit this effect. These results support the importance of the SHEP1-Cas complex in cell migration. However, SHEP1 transfected without Cas also promoted cell migration toward EGF (Fig. 9*B*). To determine whether this effect could be caused by complex formation with endogenous Cas, we co-transfected SHEP1 with a truncated form of Cas lacking the most carboxyl-terminal 133 amino acids (CasΔ133). We deleted this portion of Cas because we have established that it is sufficient to bind SHEP1 (Fig. 9*C*). CasΔ133 does not bind SHEP1 (Fig. 9*D*) but retains the other protein interaction domains, including the carboxyl-terminal Src binding domain. Therefore, CasΔ133 should act as a dominant negative by uncoupling Cas signaling complexes from SHEP1. Indeed, CasΔ133 inhibited the positive effect of SHEP1 on EGF-dependent cell migration (Fig. 9*B*). Hence, increased expression of SHEP1 is sufficient to promote chemotaxis toward EGF and the interaction of SHEP1 with endogenous Cas is important for this function. This is in agreement with our other findings that SHEP1-Cas interaction promotes Crk binding to Cas and membrane ruffling.

To examine the mechanism by which the SHEP1-Cas complex promotes cell migration, we co-transfected SHEP1 together with the CasΔSD mutant. CasΔSD significantly decreased SHEP1-mediated enhancement of cell migration toward EGF (Fig. 9*E*), confirming that the Cas substrate domain plays an important role in the effects of SHEP1 on cell migration. However, in three separate experiments, CasΔSD did not completely inhibit the effects of SHEP1 on cell migration, suggesting a contribution by additional pathways that do not involve the Cas substrate domain.

DISCUSSION

Herein, we show that SHEP1 potentiates cell migration toward EGF. By exploiting a mutation in SHEP1 that selectively impairs Cas binding, our data support a requirement for SHEP1-Cas complex formation in cell migration. The protein interface of the SHEP1-Cas complex has not been characterized, but the dramatic effects of the Y635E mutation suggest that this tyrosine of SHEP1 is a critical residue of the interface. Alignment of the SHEP1 CA-GEF domain with the related GEF domain of Sos, whose x-ray crystal structure is known (39), places tyrosine 635 in a region corresponding to the loop between helices αI and αJ of Sos. This region is located on the side opposite the Ras binding interface, consistent with the fact that the mutation does not interfere with Ras GTPase binding. However, structural information will be required to define the molecular determinants of the new type of protein interface between SHEP1 and Cas, particularly because the carboxyl-terminal half of the CA-GEF domain—which contains tyrosine 635—is not closely related to the corresponding region of Sos.

The region of Cas that binds SHEP1 consists of the 133 carboxyl-terminal amino acids. Likewise, the last 135 amino acids of Cas have been reported to be sufficient for binding another member of the SHEP family, AND-34 (40). The SHEP-binding region of Cas, which is highly conserved in the three members of the Cas family and may also mediate dimerization (41), probably folds into a domain with a three-dimensional structure similar to that of the focal adhesion-targeting domain of FAK (42, 43). It is interesting that the carboxyl terminus of Cas has also been identified as a focal adhesion targeting sequence (44). Although the SHEP proteins promote relocalization of Cas from focal adhesions to the membrane, this effect does not seem to require a direct interaction with Cas (18, 24). Thus, SHEP proteins probably do not recruit Cas to the membrane by interfering with the Cas focal adhesion targeting site or by capturing Cas through direct binding. Further studies will be required to fully elucidate how SHEP proteins cause translocation of Cas signaling complexes to the cell periphery, where they can optimally regulate cell movement.

Despite being able to trigger Cas relocalization, a truncated form of AND-34 lacking the CA-GEF domain fails to induce cell polarization, which is important for directional cell movement (24). This is in agreement with our finding that Cas binding to SHEP1 is required to induce membrane ruffling and promote EGF-dependent cell migration. Activation of Rap1 downstream of SHEP protein-Cas complexes has been implicated in Src activation, the formation of branched cell shapes, and cell migration (22, 24). Most of the data currently available, however, suggest that the SHEP CA-GEF domain does not directly activate Ras GTPases (14, 19). Rather, SHEP family proteins seem to promote activation of certain Ras and Rho proteins indirectly, through other exchange factors that in some cases function downstream of the Cas-Crk complex (22, 40, 45). Activation of the Rap1 GTPase by SHEP1 (Chat), for example, has been shown to occur downstream of Cas-Crk and the exchange factor C3G (22). The effects of SHEP1 on membrane ruffling and cell migration probably also involve Rac activation downstream of the Cas-Crk complex. Crk binds DOCK180, an exchange factor for Rac (13), and expression of the SHEP family member AND-34 has indeed been reported to increase the levels of GTP-bound Rac (45). Furthermore, Crk can enhance

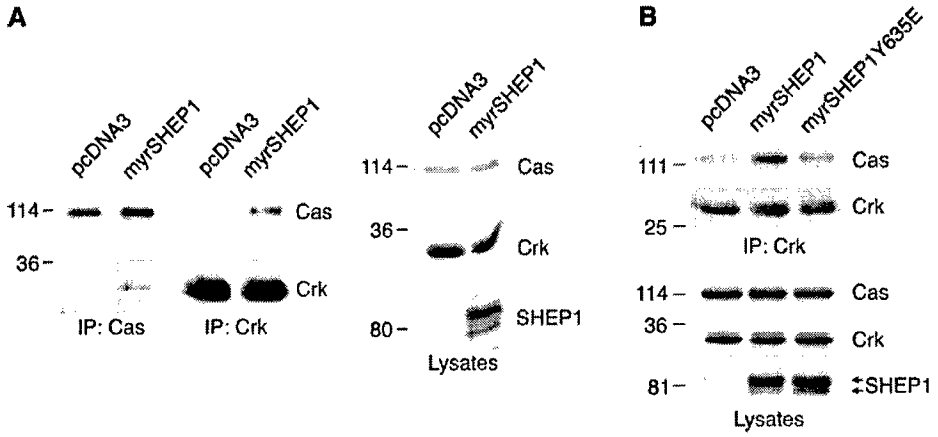

FIG. 7. **Myristoylated SHEP1 increases Cas-Crk association.** 293 cells transfected with the indicated constructs were used for immunoprecipitation (*IP*) with anti-Cas (*A*) or anti-Crk (*A* and *B*) antibodies. The immunoprecipitates were probed with anti-Crk or anti-Cas antibodies as indicated. myrSHEP1 promotes Crk binding to Cas and the Y635E mutation inhibits this effect.

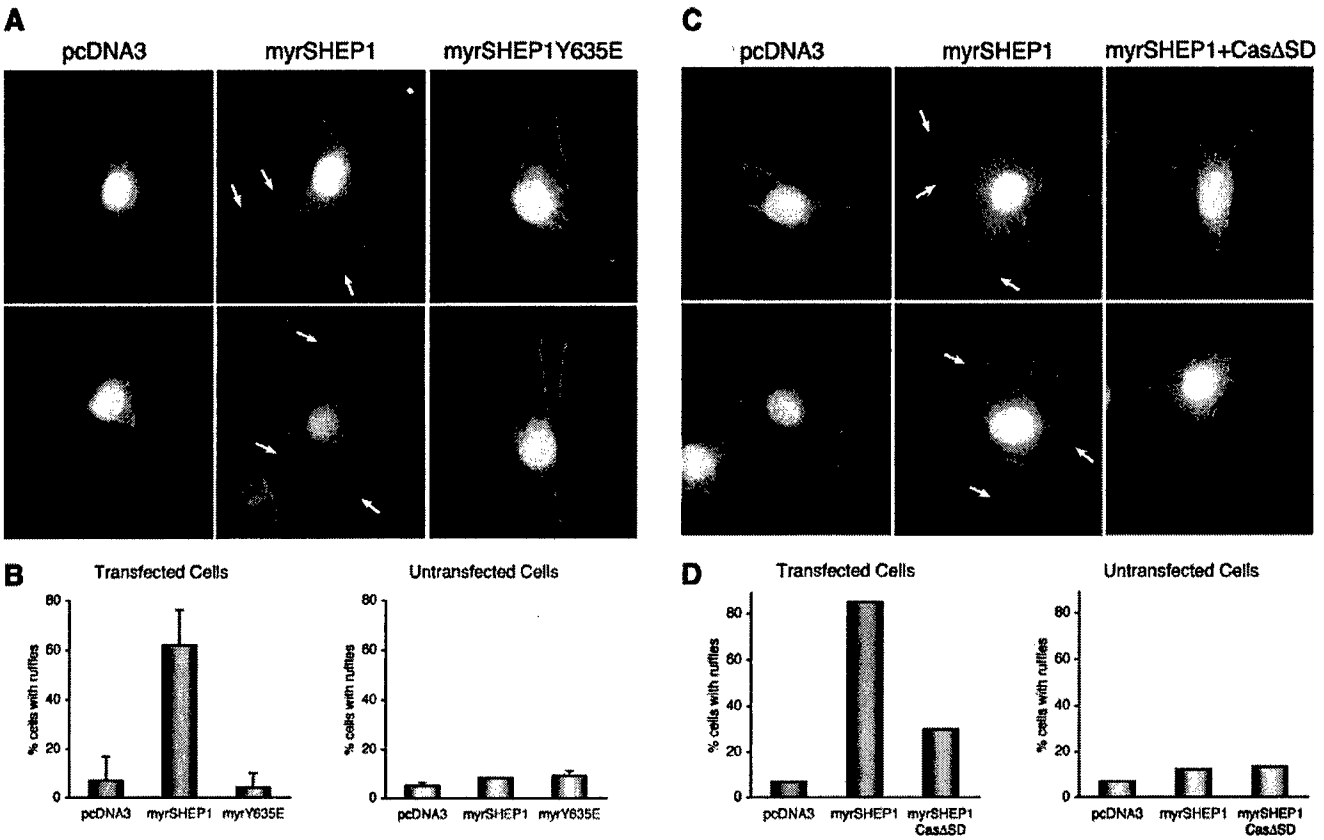

FIG. 8. **SHEP1 promotes membrane ruffling in concert with Cas.** *A* and *C*, NIH3T3 cells were transiently transfected with myristoylated SHEP1 (*myrSHEP1*), myrSHEP1 Y635E, myrSHEP1 with CasΔSD, or empty vector (pcDNA3) as a control together with EGFP (*green*) to mark the transfected cells. After trypsinization, the cells were allowed to attach for 3 h on fibronectin and then stained with phalloidin to label filamentous actin (*red*) and with DAPI to label nuclei (*blue*). Cells expressing myrSHEP1, but not the myrSHEP1 Y635E mutant deficient in Cas binding, exhibit prominent membrane ruffles (*A*). CasΔSD, which cannot bind its downstream effector Crk, inhibits the ruffling effects of myrSHEP1 (*C*). *B* and *D*, the graphs show the percentage of transfected and untransfected cells that contain ruffles, counted from populations of cells transfected with the indicated constructs. *Bars* in *B* indicate the standard deviations from two different experiments.

Rac coupling to its downstream effectors by membrane targeting, even without detectable effects on overall GTP loading (38). Consistent with a critical role of Cas-Crk signaling pathways downstream of SHEP1, we found that membrane-targeted SHEP1 promotes Cas-Crk association and that mutant Cas lacking the Crk-binding domain decreases the positive effects of SHEP1 on membrane ruffling and EGF-dependent cell migration.

SHEP proteins nevertheless have the ability to directly bind Ras family GTPases. For example, SHEP1 binds R-Ras, Rap1 (14), and, as we show here, Rap2. These Ras proteins are known to regulate integrin-mediated adhesion (19, 46, 47), and SHEP1 may serve to sequester them or recruit them to specific subcellular locations. Mutations that selectively disrupt the association of SHEP1 with Ras proteins but not with Cas would help to further clarify the function of Ras GTPase binding to SHEP proteins.

Activation of JNK may also play a role in cell migration downstream of the SHEP1-Cas complex. Both SHEP1 and Cas promote JNK activation when overexpressed in cells (16, 18,

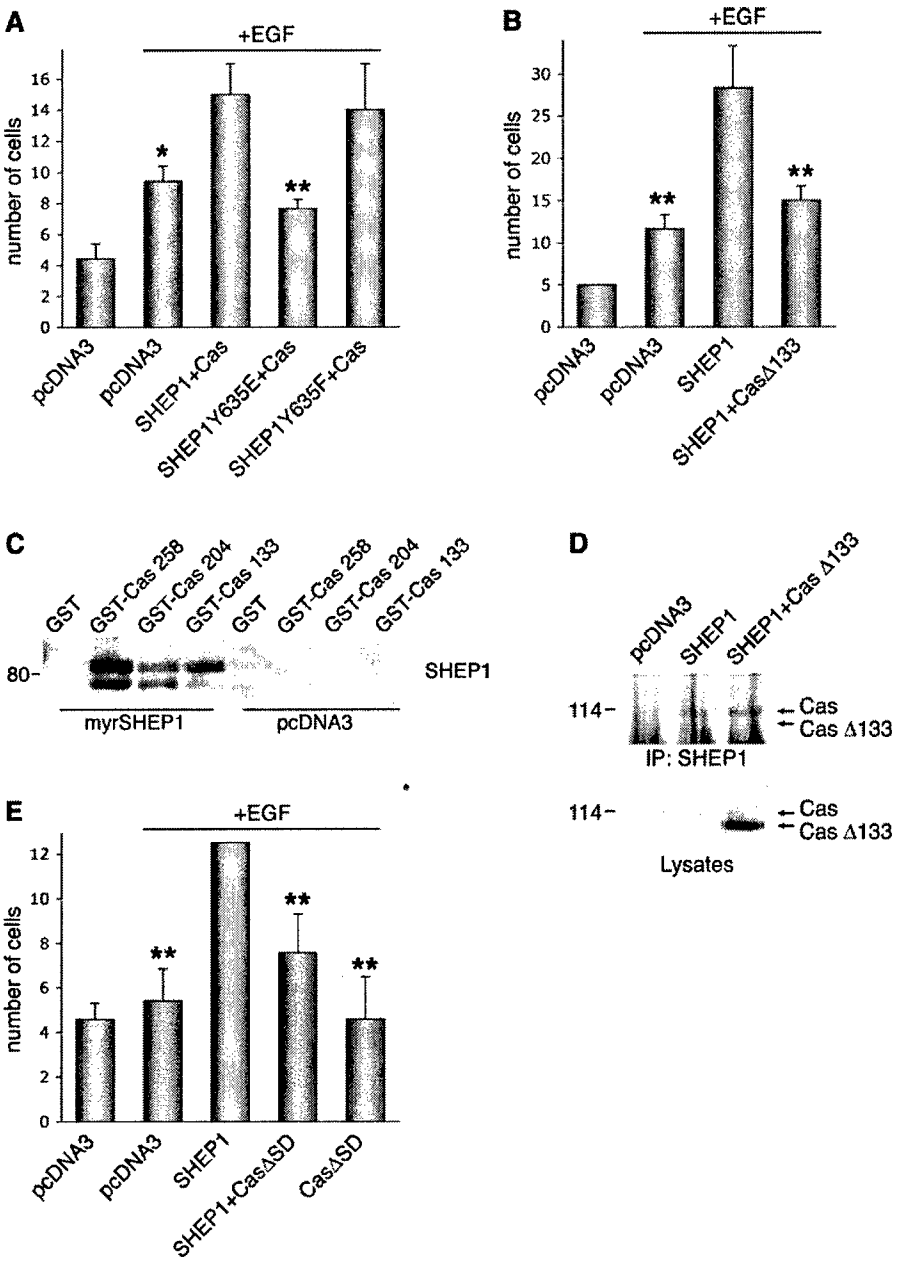

FIG. 9. **SHEP1 promotes cell migration toward EGF in a Cas-dependent manner.** *A*, SHEP1 and SHEP1 Y635F co-transfected with Cas promote cell migration toward EGF, whereas SHEP1 Y635E does not. Equal numbers of COS cells transfected with the indicated constructs and EGFP were seeded on Transwell filters coated on both sides with fibronectin. The cells were allowed to migrate through the filters toward EGF in the lower chamber or, as a control, in the absence of EGF. The histogram shows the number of transfected (EGFP-positive) cells that migrated through the filters in 5 h. *Bars* indicate the standard deviations from three measurements corresponding to three different filters. The EGF-treated samples were all compared with SHEP1+Cas by one-way analysis of variance and Dunnett's post-hoc test; *, $p < 0.05$ and **, $p < 0.01$. *B*, SHEP1 transfected alone promotes cell migration toward EGF, and a mutant form of Cas that does not bind SHEP1 inhibits this effect. The experiment was carried out as in *A*, except that the cells were counted after 4 h of migration. The EGF-treated samples were all compared with SHEP1 by one-way analysis of variance and Dunnett's post-hoc test; **, $p < 0.01$. *C*, the C-terminal 133 amino acids of Cas are sufficient to bind SHEP1. GST fusion proteins containing the carboxyl-terminal 258, 204, and 133 amino acids of Cas were immobilized on glutathione agarose and incubated with lysates of 293 cells transfected with myrSHEP1 or pcDNA3 as a control. Bound proteins were probed by immunoblotting with anti-SHEP1 antibodies. *D*, CasΔ133, which lacks the C-terminal 133 amino acids, does not bind SHEP1. SHEP1 immunoprecipitates from COS cells transfected with the indicated constructs were probed with anti-Cas antibodies. Although detected at much higher levels than endogenous Cas in the lysates, CasΔ133 cannot be detected in the immunoprecipitates. *E*, the experiment was carried out as in *B*, except that cell numbers were adjusted for transfection efficiency because of differences in transfection efficiencies between samples. The EGF-treated samples were compared with SHEP1 by one-way analysis of variance and Dunnett's post-hoc test; **, $p < 0.01$. CasΔ133, Cas lacking the C-terminal 133 amino acids; CasΔSD, Cas lacking the substrate domain.

29), and evidence suggests that they do so through a shared signaling pathway (23). It is noteworthy that JNK binds to Crk (48) and could therefore be localized at sites of Cas-Crk interaction. Furthermore, recent data show that JNK promotes cell migration in various cell types by phosphorylating serine 178 of paxillin (49).

We found that overexpression of SHEP1 is sufficient to enhance EGF-dependent migration. AND-34 has also been reported to enhance cell migration toward serum and haptotaxis toward fibronectin, but only when Cas is also overexpressed (24). The different cell types used may explain this discrepancy. The relative levels of endogenous SHEP proteins and Cas in a particular cell type may determine whether increased expression of a SHEP protein can promote cell migration through a pathway involving Cas. In addition, the relative levels of Cas and Src kinases may also be important (50). AND-34 required Cas to promote migration of C3H10T1/2–5H murine fibroblasts, which contain highly elevated levels of Src, whereas SHEP1 promoted migration of COS cells with normal Src levels. Src overexpression may also contribute to the pronounced increase in phosphorylation of Src substrates observed after transfecting AND-34 and Cas in COS cells (24). Indeed, we did not detect increased tyrosine phosphorylation of the Src substrate paxillin in COS cells transfected only with myrSHEP1 (data not shown). However, myrSHEP1 increased Cas phosphorylation by Src, possibly by promoting relocalization of Cas near Src and/or Src activation in a restricted microenvironment.

EGF receptor-mediated chemoattraction has been shown to require Cas (11, 51) and, as we show here, interaction with SHEP proteins plays an important role on the positive effects of Cas on EGF-dependent cell migration. The EGF receptor can also promote neurite extension through a pathway involving Src, the Cas family protein Sin, and Crk (52). In contrast, the EphB2 receptor promotes repulsive migratory responses and neurite retraction (34, 53, 54) and, as we found, causes dissociation of SHEP1 from Cas. An intriguing possibility is that differential regulation of the SHEP1-Cas complex may contrib-

ute to the positive or negative effects of certain receptor tyrosine kinases on cell movement and neurite extension. The EGF receptor does not disrupt the association between SHEP proteins and Cas and may even increase it in some cases (16). In contrast, the effects of the Y635E mutation suggest that phosphorylation of this tyrosine downstream of EphB2 inhibits Cas binding and signaling downstream of the SHEP-Cas complex. However, additional regulatory mechanisms must also contribute to SHEP-Cas dissociation because EphB2 also disrupts the complex between Cas and the SHEP1 Y635F mutant, which lacks the tyrosine 635 phosphorylation site (data not shown). It is noteworthy that tyrosine 635 is conserved in AND-34 but not Nsp1, which has phenylalanine at this position, indicating that Nsp1 cannot be regulated by phosphorylation at this site. It will be interesting to determine whether regulation of SHEP-Cas coupling influences the biological activities of other receptor tyrosine kinases that use Cas-Crk signaling (55).

Although EGF, insulin, and integrin-mediated cell adhesion to fibronectin induce tyrosine phosphorylation of SHEP family members, the significance of these phosphorylation events has remained unknown (16, 17). In addition to tyrosine 635 in the CA-GEF domain, our mass spectrometry experiments identified tyrosines 121 and 126 in the SHEP1 SH2 domain as phosphorylation sites downstream of EphB2. Tyrosine 121 was also identified as a SHEP1 phosphorylation site downstream of Bcr-Abl in myeloid leukemia cells (56). Phosphorylation of tyrosine 121, which is in strand βE of the SH2 domain, may regulate the binding affinity and specificity of the SHEP1 SH2 domain—like phosphorylation of the similarly located tyrosine in the Src and Lck SH2 domains (57, 58). Therefore, phosphorylation of the SHEP1 SH2 domain may modulate the localization of SHEP1-Cas complexes during cell migration.

Previous work has identified two EGF-dependent tyrosine phosphorylation sites in the SHEP family protein Nsp1 (16). One of these phosphorylation sites is in a position similar to that of tyrosine 142 of SHEP1, whereas the other is not conserved in SHEP1. We did not detect phosphorylated peptides containing tyrosine 142 by MALDI-TOF mass spectrometry in cells expressing activated EphB2. However, not all peptides are detectable with this approach. We identified tyrosines 170 and 175 in the proline/serine-rich region as phosphorylation sites downstream of EphB2 and detected additional peaks corresponding to peptides containing other SHEP1 tyrosines (Table I), but their identity needs to be confirmed because peptide assignments based on a single peak are unreliable. In addition to regulating SHEP-Cas coupling and SH2 domain interactions, tyrosine phosphorylation of SHEP1 may also initiate signaling pathways that are independent of the Cas-Crk complex by recruiting signaling molecules containing SH2 and PTB domains to SHEP1. There is some evidence that SHEP family members may use alternative signaling pathways in addition to Cas-Crk. For example, AND-34 has recently been reported to promote activation of the Rho family GTPase Cdc42 through an unknown pathway (40). Further studies with SHEP1 phosphorylation site mutants will be required to determine whether tyrosine phosphorylation of SHEP1 may play a role in Cdc42 activation and cell migration and to identify the sites phosphorylated by Src downstream of the EGF receptor.

The SHEP and Cas families are ubiquitously expressed (14, 16–18),[2] implying an essential role in normal cellular physiology. Besides playing a role in pathways that regulate cell migration, proteins of the SHEP and Cas families have several other similar functions that may depend upon their association and shared downstream signaling pathways. For example, AND-34 and Cas can up-regulate cyclin-D1 expression and overcome the effects of the cytostatic agent tamoxifen in estrogen-dependent breast cancer cells (15, 21, 45). In addition, SHEP1 (Chat) and Cas-L promote JNK activation and interleukin-2 production in T-cells (23). Therefore, elucidating the role of SHEP-Cas complexes is important for understanding basic cellular functions controlling cell migration, proliferation, and immune function. In addition, it may also help clarify how defects in cell migration cause pathologies such as tumor cell invasion and metastasis and how persistent cell proliferation under adverse conditions can lead to tumor cell growth and resistance to chemotherapy.

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[2] V. S. Vervoort and E. B. Pasquale, unpublished data.

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